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## Box Patent Application

Assistant Commissioner for Patents  
Washington, DC 20231

Presented for filing is a new original patent application of:

Applicant: IKUO MATSUI, KAZUHIKO ISHIKAWA, HIROYASU  
ISHIDA, YOSHITSUGU KOSUGI  
Title: THERMOPHILIC ENZYMES HAVING  $\beta$ -GLYCOSIDASE  
ACTIVITY

Enclosed are the following papers, including all those required to receive a filing date  
under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	35
Claims	2
Abstract	1
Drawing(s)	7

### Enclosures:

- Nucleotide and/or amino acid sequence listing including:  
Computer readable copy.  
Paper copy, 7 pages.  
Verification statement, 1 pages.
- A certified copy of the priority application will be filed at a later date.
- Postcard.

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Under 35 USC §119, this application claims the benefit of a foreign priority application filed in Japan, serial number 222866/1998, filed 8/6/98.

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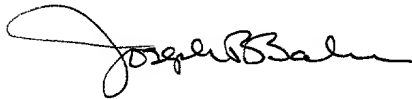
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Respectfully submitted,



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Enclosures  
98959 LJ1

# APPLICATION FOR UNITED STATES PATENT

in the name of

**IKUO MATSUI, KAZUHIKO ISHIKAWA, HIROYASU  
ISHIDA, YOSHITSUGU KOSUGI**

of

**Director-General of  
Agency of Industrial Science and Technology**

for

**THERMOPHILIC ENZYMES HAVING  
 $\beta$ -GLYCOSIDASE ACTIVITY**

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## SPECIFICATION

THERMOPHILIC ENZYMES HAVING  $\beta$ -GLYCOSIDASE ACTIVITY

## BACKGROUND OF THE INVENTION

The present invention relates to a thermophilic enzyme having  $\beta$ -glycosidase activity. More particularly, the present invention relates to a thermophilic enzyme having  $\beta$ -glycosidase activity derived from a hyperthermophilic bacterium belonging to the genus *Pyrococcus*.

$\beta$ -Glycosidases are useful for hydrolysis of saccharides, DNA sequencing, conformational analysis of glycoproteins and glycolipids, and enzymatic synthesis of oligosaccharides and heterosaccharides with high optical purities. The catalytic reaction of  $\beta$ -glycosidases with substrates is specific with respect to the types of the monosaccharides constituting the substrates, and the optical isomerism and the position of the glycosidic linkage to be cleaved in the substrates.  $\beta$ -Glycosidases are also useful for the modification of sugar chains and the synthesis of oligosaccharides and polysaccharides retaining their optical stereoisomerism, as well as the synthesis of heterosaccharides (e.g., biosurfactants) due to their ability to transfer a glycoside group into a primary, secondary or tertiary alcohol. Hitherto, various types of  $\beta$ -glycosidases with different substrate-specificities have been found in bacteria and plants. However, since many of such  $\beta$ -glycosidases are derived from mesophilic organisms, they are poor in thermal

resistance, and consequently are unsuitable for use in synthetic reactions under such extreme conditions that organic solvents are used simultaneously.

If a thermophilic  $\beta$ -glycosidase active in organic solvents is found, this can be used as a biocatalyst to develop a new procedure for synthesizing a heterosaccharide with high optical purity. In this procedure, the reverse hydrolytic reaction (i.e., synthetic reaction) is utilized which predominately occurs in the presence of an organic solvent. Under the circumstances, a novel  $\beta$ -glycosidase which is active under extreme conditions has been strongly demanded.

#### SUMMARY OF THE INVENTION

An object of the present invention is to provide a thermophilic enzyme with  $\beta$ -glycosidase activity.

For solving the above-mentioned problems, the present inventors focused on hyperthermophilic bacteria capable of growing within the temperature range from 90 to 100° C. As a result, they have found a gene that is assumed to encode a protein having  $\beta$ -glycosidase activity from its nucleotide sequence. The inventors have succeeded in the production of an enzyme from the gene by introducing the gene into *Escherichia coli* cells to transform the cells and then producing the enzyme from the transformants, which enzyme was confirmed to be stable at high temperatures (90° C or higher) and to have  $\beta$ -glycosidase activity. This success leads the accomplishment of the invention.

That is, the present invention provides a thermophilic

enzyme having  $\beta$  -glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added. The number of the amino acid residue which may be deleted, replaced or added in the amino acid sequence of SEQ ID NO: 2 is not particularly limited as long as the  $\beta$  -glycosidase activity is retained, but preferably from 1 to 30, and more preferably from 1 to 18. It is preferable to delete, replace or add an amino acid residue or residues present in any of the regions of amino acid residues 78-86, 154-171 and 1-423. The enzyme preferably has an optimum temperature of 100° C or higher.

The present invention also provides a DNA which is capable of hybridizing to the nucleotide sequence of SEQ ID NO: 1 or to the complement thereof under such conditions that the hybridization is carried out in 6xSSC and 50% formamide at 42 °C and the washing process is carried out in 6xSSC and 40% formamide at 25 °C, and which encodes a thermophilic enzyme having  $\beta$  -glycosidase activity. These conditions are of low stringent. A moderate stringent conditions are such that the hybridization is carried out in 6xSSC and 40% formamide at 42 °C and the washing process is carried out in 1xSSC and 0% formamide at 55 °C. A high stringent conditions are such that the hybridization is carried out in 6xSSC and 30% formamide at 42 °C and the washing process is carried out in 0.1xSSC and 0% formamide at 62 °C. The DNA may encode a thermophilic enzyme which comprises the amino acid sequence of SEQ ID NO: 2 in which of one or a plurality of amino acid residues may be deleted,

replaced or added and which has  $\beta$  -glycosidase activity.

The present invention further provides a recombinant vector containing the DNA therein, a host cell transformed with the recombinant vector, and a process for producing the enzyme comprising culturing a host cell transformed with an expression vector containing a DNA encoding the enzyme and then collecting the enzyme from the resultant culture. Using this process, the mass production of the enzyme becomes possible.

The present invention further provides a process for the hydrolysis of a  $\beta$  -glycoside having a long alkyl chain at the reducing end, with a thermophilic enzyme having  $\beta$  -glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added. The long alkyl chain may be an alkyl group having carbon atoms of 8 or more. The hydrolysis may be carried out at a temperature of 85° C or higher, and preferably 100° C or higher.

This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 10-222866, which is a priority document of the present application and incorporated herein by reference in its entirety.

The above and other objects, effects, features and advantages of the present invention will become more apparent from the following description of embodiments thereof taken in conjunction with the accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of the Triton X-100 concentration on the His-BGPh Activity. The standard of 100% was defined as the activity at 0.1% Triton X-100.

Figure 2 shows thermostability of His-BGPh at 90°C. Triton X-100 at 0.1% was present in the reaction mixtures. The standard of 100% was defined as the activity without heating.

Figure 3 shows optimum pH of the activity for His-BGPh. The OD405 indicates the amount of released p-Nph group in acetate buffer (square) and phosphate buffer (circle). The closed symbols correspond to the activity of BGPh and open symbols correspond to the activity of His-BGPh. For these measurements, equal amounts of BGPh and His-BGPh were used because the heated suspension I (BL21(DE3)/pET-11a/BGPh or BL21(DE3)/pET-15b/BGPh) was estimated to contain the same amount of each induced protein by quantification using SDS-PAGE analysis.

Figure 4 shows temperature dependency of BGPh. Optimum temperature was determined by the plots of enzymatic activity (OD405 nm change) against reaction temperature. An Arrhenius plot of the data is given in the inset.

Figure 5 shows aligned amino acid sequences of five  $\beta$  - glycosidases from hyperthermophilic archaea. The abbreviations of the sources of the enzymes are : BGPh,  $\beta$  - glycosidase from *P. horikoshii*; BMPH, a  $\beta$  - mannosidase gene homolog from *P. horikoshii* (8, 9); BGPf,



$\beta$  - glucosidase from *P. furiosus* (17); BMPf,  $\beta$  - mannosidase from *P. furiosus* (17);  $S\beta$  - gly,  $\beta$  - glycosidase from *Sulfolobus solfataricus* (18). The conserved residues, identified automatically by the GeneWorks program, are shown in the open boxes. The reversed open triangles indicate the location of the nucleophile (E324) and the putative acid/base catalyst (E155 and H111) with R75 in the spatial proximity of the nucleophile of BGPh. The arrow shows the prominent deletion of more than 30 residues found in BGPh.

Figure 6 shows illustrated location of the four hydrophilic edges on the tetragonal structure of  $S\beta$  - gly (30) and the four hydrophobic areas exposed by removing the hydrophilic loops forming the edges. (A) The tetragonal arrangement with the hydrophilic edges (blue). (B) The tetragonal arrangement with a hydrophobic surface (red) created by the deletion of the hydrophilic loops, shielding barrel helices 3 and 4 from solvent.

Figure 7 shows a comparison of hydropathy profiles between BGPh and  $S\beta$  - gly. The panel (A) shows the hydropathy profile of BGPh and panel (B) shows the hydropathy profile of  $S\beta$  - gly. The arrows indicate the corresponding residue numbers. Two hydrophobic clusters are observed in BGPh but not in  $S\beta$  - gly.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention will be described specifically below.

The enzyme according to the present invention is a

thermophilic enzyme having  $\beta$ -glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added. The enzyme comprising an amino acid sequence of SEQ ID NO: 2 and having  $\beta$ -glycosidase activity is derived from a sulfur-metabolizable thermophilic archaeon *Pyrococcus horikoshii* (the accession number: JCM 9974). One example of the processes for producing the enzyme is described below.

First, cells of *Pyrococcus horikoshii* are cultured and then chromosomal DNA was prepared therefrom. The chromosomal DNA is digested with restriction enzyme(s) to give fragments, and a genomic DNA library is constructed using the fragments. Clones which cover the chromosome of *Pyrococcus horikoshii* are selected and aligned. The aligned clones are sequenced and a gene encoding a  $\beta$ -glycosidase is identified. The nucleotide sequence of the gene encoding  $\beta$ -glycosidase is depicted in SEQ ID NO: 1. The gene is amplified by the PCR method and then extracted. The extracted gene is inserted into an expression plasmid suitable for protein production (e.g., pET11a or pET15b). The resultant recombinant plasmid is introduced into cells of a host (e.g., *Escherichia coli*), from which the enzyme can be produced. The produced enzyme is isolated and purified by heating and then subjecting to column chromatography.

As a result, it is revealed that the purified enzyme is a protein having a molecular weight of about 45,000 Da and capable of hydrolyzing  $\beta$ -glycosides. When the enzyme

is treated in 50 mM phosphate buffer (pH 6.0) containing 250 mM NaCl at 95° C for 1 hour, its activity is retained at the level of 80% based on the initial level. The enzyme has an optimum pH of pH 6.0 and an optimum temperature of 100° C or higher in terms of the enzymatic activity.

Variants of the enzyme, that is, thermophilic enzymes comprising deletion, replacement or addition of one or a plurality of amino acid residues in the amino acid sequence of SEQ ID NO: 2 and having  $\beta$ -glycosidase activity, may be prepared by any known techniques, such as site-specific mutagenesis and the PCR method.

The enzymes of the present invention can be used for hydrolysis of saccharides, DNA sequencing, conformational analysis of glycoproteins and glycolipids, synthesis of origosaccharides and heterosaccharides with high optical purities, and the like.

#### DEPOSIT OF MICROORGANISM

A transformant designated "E. coli BL21 (DE3) pET15b/Gly2M" which is E. coli BL21 (DE3) transformed with an expression vector containing a  $\beta$ -glycosidase gene (pET15b/Gly2M) was deposited under the terms of the Budapest Treaty on July 27, 1999 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaragi-ken, Japan) under Accession No. FERM BP-6800.

The following examples are given as more specific

illustration of the invention. It should be understood, however, that the invention is not limited to the specific details set forth in the examples.

## EXAMPLES

Abbreviations: BGPh,  $\beta$ -glycosidase from *P. horikoshii*; BPh, a  $\beta$ -mannosidase gene homolog from *P. horikoshii*; BGPf,  $\beta$ -glucosidase from *P. furiosus*; BMPf,  $\beta$ -mannosidase from *P. furiosus*; S $\beta$ -gly,  $\beta$ -glycosidase from *Sulfolobus solfataricus*; Amp, ampicillin; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; His-BGPh, BGPh with His-tag; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBBR, Coomassie Brilliant Blue R; X-Glu, 5-bromo-4-chloro-3-indolyl- $\beta$ -glucopyranoside; p-Nph- $\beta$ -D-Glcp, p-nitrophenyl  $\beta$ -D-glucopyranoside; LA- $\beta$ -D-Glcp,  $\beta$ -D-glucopyranosides with long alkyl chains.

## MATERIALS AND METHODS

Chemicals - The pET-11a vector and ultracompetent *E. coli* XL2-Blue MRF' cell were purchased from Stratagene. The pET-15b vector and *E. coli* strain BL21 (DE3) were obtained from Novagen. Vent DNA polymerase was purchased from New England Biolabs. Restriction enzymes were purchased from Promega and Toyobo (Osaka, Japan), and were used according to the manufacturers' recommendations. Ultrapure deoxynucleotide solution (dNTPs) was obtained from Pharmacia Biotech. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was from Takara Shuzo (Otsu, Shiga, Japan).

### *Cloning of Genes and Construction of Expression Vector*

- The genome of *P. horikoshii* was sequenced using the method of Kaneko et al. (10). Standard cloning techniques were used throughout. The expression vectors pET-11a and pET-15b were double-digested by the restriction enzymes Nde I and BamH I and the resulting 5.7Kbp fragment was purified with a QIAquick Gel Extraction Kit (QIAGEN). The gene coding  $\beta$  - glycosidase (BGPh) was amplified by the PCR method using the following two primers: upper primer, TAAGAAGGAGATATACATATGCCGCTGAAATTCCCGGAAATGTTTCTCTTTGGT ACC (SEQ ID NO: 3); lower primer, TTTACTGCAGAGAGGATCCCTAATCCTAAAGTTGAAGTTCTGGTAG (SEQ ID NO: 4). The PCR product was cloned into expression vectors pET-11a and pET-15b using NdeI and BamHI sites.

The digested 1.3 Kbp fragment coding BGPh was purified and ligated to the insertion sites of the pET-11a and pET-15b vectors. Ultracompetent *E. coli* XL2-Blue MRF' cells were transformed with the recombinant molecule. Transformants were screened on 2 xYT plates containing 50 mg/ml of ampicillin (Amp) incubated at 37°C overnight. The transformant colonies were propagated in 5 ml 2 x YT +Amp medium at 37°C overnight and the vectors pET-11a/BGPh and pET-15b/BGPh were purified after centrifugation using a Mini Plasmid Kit (QIAGEN). The pET-11a/BGPh and pET-15b/BGPh were double-digested with NdeI and BamHI and the insert length was checked using agarose gel electrophoresis. The absence of additional mutations within the coding region of BGPh was verified by sequencing on an Applied Biosystems 373A DNA sequencer (Taq DyeDeoxy

Terminator Cycle Sequencing Kit, PerkinElmer).

#### *Overexpression and Purification of Recombinant Protein*

The *E. coli* strain BL21 (DE3) was transformed with the pET-11a/BGPh plasmid to express mature BGPh and pET-15b/BGPh plasmid to express His-tagged BGPh. The transformant colony was propagated as seed culture in 200 ml 2 x YT +Amp medium at 37°C overnight. An inoculate of 40 ml seed culture was inoculated to 2 l of 2 x YT +Amp medium. The transformant was induced at OD<sub>600</sub>=1 with 1 mM IPTG for 4 h. The induced cells were collected by centrifugation and stored at -20°C.

The frozen cells (7 g) were thawed and mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5) and 5.6 ml of 10% Triton X-100, resulting in a final concentration of 2.5%. The cell suspension was heated at 85°C for 10 min, then centrifuged at 5000 x g for 20 min. The supernatant was collected and stored at 4°C. The cell pellet was mixed with the same volume of the buffer and Triton X-100 and heated again. The heated sample was centrifuged at 25000 x g for 20 min. The combined supernatant was mixed with 1 mg of bovine DNase 1 (Sigma) and incubated at 37°C for 30 min. The supernatant was heated at 85°C for 10 min, then centrifuged at 25000 x g for 20 min to remove the inactivated DNase.

The solubilized recombinant BGPh with His-tag (His-BGPh) was subjected to affinity chromatography with Ni-conjugated Sepharose, using a stepwise elution from 5 mM to 1 M imidazole in 20 mM Tris-HCl (pH 8.0) with 0.5 M NaCl solution (His-bind Buffer Kit, Novagen) containing 0.1% Triton X-100. BGPh was eluted with 100 mM imidazole

with 0.1% Triton X-100. The enzyme samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11); a low molecular weight electrophoresis calibration kit, purchased from Pharmacia Biotech, was also run. For SDS-PAGE (PhastGel, 10-15%), the enzyme sample (5 ml) was mixed with SDS sample buffer (5 ml), boiled for 5 min, mixed with marker dye (1 ml) and applied to the gel in 1 or 4 ml aliquots. Following electrophoresis, protein was detected by Coomassie Brilliant Blue R (CBBR) staining according to the manufacturer's recommendation. The His-tagged protein was detected with QIAexpress Detection System (QIAGEN) after blotting onto a nitrocellulose membrane (Pharmacia Biotech).

*Cellular Localization of the Activity* - Localization of the BGPh activity in E. coli transformant cells (BL21(DE3)/pET-11a/BGPh or BL21(DE3)/pET-15b/BGPh) was examined by fractionation of the cell components. The cell membrane was isolated as follows: 7 g of the induced cells, which were frozen at  $-20^{\circ}\text{C}$ , were thawed and mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5). The cell suspension (suspension I) was sonicated with a Sonifier 250 (Branson) for 4 min at an output control level of 4 and at 30% duty cycle. The sonicated sample was centrifuged at  $9,000 \times g$  for 10 min to remove cell debris, then the supernatant (12 ml) was ultracentrifuged at  $100,000 \times g$  for 1 h to separate the membrane fraction (1 ml) from the supernatant. The enzyme reactions were carried out at  $90^{\circ}\text{C}$  for 15 min in a solution (200 ml) containing 1.2 mM 5-bromo-4-

chloro-3-indolyl- $\beta$  - glucopyranoside (X-Glu) and 5 ml of each fraction, as the enzyme source, in 50 mM phosphate buffer (pH 6) with 0.3 M NaCl. After the reaction, the solution was cooled in ice and diluted with 1 ml of water; the absorbance at 620 nm was immediately measured. As a control, the assay reactions were performed under the same conditions but without X-Glu to subtract the turbidity derived from each fractionated sample.

To analyze the solubilizing effect of Triton X-100, suspension I was also heated with and without 2.5% Triton X-100 at 85°C for 10 min and the supernatant was obtained by centrifugation at 15,000 x g for 10 min. The activity of the supernatants was measured using X-Glu as shown above.

*Dependence of the BGPh Activity on Triton X-100* - The enzyme reactions were carried out at 98°C for 20 min in a solution (200 ml) containing 3 mM p-Nph- $\beta$  - D-Glcp (a p-nitrophenyl saccharide) and 57.5 pM of the purified His-BGPh in 50 mM phosphate buffer (pH 6) with Triton X-100 and 0.1 M NaCl. The concentration of Triton X-100 in the reaction solution was varied from 0.1% to 0.00002%. The reaction was terminated by the addition of 1 M Na<sub>2</sub>CO<sub>3</sub> (1 ml), then centrifuged at 15,000 x g for 10 min. The concentration of the p-Nph group in the supernatant was quantified by measuring the absorbance at 400 nm.

*Measurement of the Kinetic Parameters* - The enzyme reactions were carried out at 90°C in a solution (200 ml) containing the substrate and the purified His-BGPh in 50 mM phosphate buffer (pH 6) with 0.1% Triton X-100 and 0.3 M NaCl. For the hydrolysis of p-nitrophenyl (p-Nph)  $\beta$



- D-saccharides, the reaction was terminated by the addition of 1 M Na<sub>2</sub>CO<sub>3</sub> (1 ml), then centrifuged at 15,000 x g for 10 min. The concentration of the p-Nph group in the supernatant was quantified by measuring the absorbance at 400 nm. For the hydrolysis of  $\beta$  - D-glucoside, the released glucose was analyzed with a Glucose C-II Test kit (Wako Pure Chemicals, Japan). Initial velocities were obtained directly from the initial slopes of the time course plots. The K<sub>m</sub> and k<sub>cat</sub> values were calculated using the Michaelis-Menten equation and the least squares method (12). The subsite affinity for a long alkyl chain was determined using the method reported previously (13-15) on the basis of the subsite theory (16).

*Optimum Temperature and Optimum pH* - The optimum temperature was measured as follows: the assay mixture (200 ml), which contained 3 mM p-nitrophenyl  $\beta$  - D-glucopyranoside (p-Nph- $\beta$  - D-Glcp) in 150 mM citrate buffer (pH 5.0) and 1 ml of suspension I (BL21(DE3)/pET-11a/BGPh), was heated at 85°C for 10 min. The enzyme reactions were carried out in duplicate at temperatures ranging from 50 °C to 100 °C for 30 min. Optical density measurements at A<sub>405</sub> were performed as described for the enzyme assays.

To determine the optimum pH, the assay mixture (200 ml), which contained 1 ml of heated suspension I (BL21(DE3)/pET-11a/BGPh or BL21(DE3)/pET-15b/BGPh) and p-Nph- $\beta$  - D-Glcp (3 mM) in 139 mM buffer systems, was heated at 90 °C for 30 min. The pH of the reaction mixtures ranged from 3.9 to 5.5 in sodium acetate buffer and from 5.5 to 7.99 in phosphate buffer. Optical density measurements

at A405 were performed as described for the enzyme assays.

*Thermostability* - The His-BGPh solutions (29 nM) in 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl and 0.1% Triton X-100 were heated in sealed Eppendorf tubes at 90°C in various increments up to 24 h. The heated enzymes were assayed in duplicate in phosphate buffer (pH 6.0) at 90°C for 20 min as described for the determination of optimum temperature.

*Sequence Alignment, Phylogenetic Tree, and Hydropacy Profile* - Sequence alignment of  $\beta$  - glycosidases was performed using the GeneWorks program (IntelliGenetics, Inc.) based on a PAM-250 scoring matrix. The enzymes of interest were:  $\beta$  - glycosidase (BGPh) studied in this paper and  $\beta$  - mannosidase (BMPh) from *P. horikoshii* (8, 9),  $\beta$  - glucosidase (BGPf) and  $\beta$  - mannosidase (BMPf) from *P. furiosus* (17), and  $\beta$  - glycosidase ( $S\beta$  - gly) from *Sulfolobus solfataricus* (18). Phylogenetic trees for the same sequences were constructed using the GeneWorks program based on the unweighted pair group method with an arithmetic mean (19). Each hydropacy profile was analyzed with DNASIS-Mac v2.0 software based on the Kyte and Doolittle method (20).

## RESULTS AND DISCUSSION

*Localization of the Activity in E. coli Membrane* - The intracellular localization of His-BGPh was examined (Table I).

Table I. Cellular localization of the activity. The transformant *E. coli* BL21(DE3)/pET15b/BGPh cells were used for this experiment. The enzyme reaction were performed at 90°C and pH 6 for 15 min using X-Glu as substrate, and then A<sub>620</sub> was measured as shown in "MATERIALS AND METHODS".

Cell fractions	Activity after each treatment (A <sub>620</sub> )				
	Sonication	Non-heated	Heated	Non-heated with 2.5% Triton X-100	Heated with 2.5% Triton X-100
Suspension I	0.585	0.585	0.567	0.485	0.428
Supernatant at 9,000 xg	0.112	ND	ND	ND	ND
Supernatant at 15,000 xg	ND	0.008	0.005	0.107	0.255
Supernatant at 100,000 xg	0.010	ND	ND	ND	ND
Fraction precipitated at 100,000 xg	0.478	ND	ND	ND	ND

ND; not determined.

The induced cells were disrupted by sonication and centrifuged to separate the cell components. The membrane fraction was precipitated by ultracentrifugation at 100,000 x g from the supernatant recovered by centrifugation at 9,000 x g. The activity was present in the membrane fraction whereas no activity was detected in the soluble fraction after the ultracentrifugation. His-BGPh was solubilized from the cell suspension (suspension I) by heating with a detergent, Triton X-100; the enzyme was not solubilized by heating without Triton X-100. The solubilizing efficiency with Triton X-100 was elevated by heating up to 85°C, whereas only 22% of the activity was extracted at room temperature. The best condition for the solubilization was 2.5% Triton X-100 at 85°C for 15 min. The native-type BGPh was also solubilized under the same condition as His-BGPh (data not shown); however, the denaturation with 8 M urea and the renaturation by direct dilution with buffer had no effect on the solubilization of the activity (data not shown). These

facts strongly indicate that BGPh is a thermostable membrane protein solubilized by Triton X-100.

His-BGPh was purified by one-step affinity chromatography using Ni-conjugated Sepharose. Since the recovery of the active enzyme was decreased to a few percent by the elimination of Triton X-100 from the chromatographic washing and elution buffers, the presence of Triton X-100 in the buffer system was essential for the stabilization of BGPh.

As shown in Figure 1, the activity of BGPh was dependent on the concentration of Triton X-100. At 0.00002% Triton X-100, the activity decreased to 10% of that with 0.1% Triton X-100. Furthermore, BGPh was stabilized in the presence of 0.1% Triton X-100: the half-life of the activity was 15 h at 90°C and pH 6.0 (Fig. 2). These facts also suggest that BGPh is the membrane protein.

*The Substrate Specificity of BGPh* - For BGPh both with or without His-tag, the optimum pH was 6.0 (Fig. 3) and the optimum temperature was over 100 °C (Fig. 4). The substrate specificity of His-BGPh was examined using p-Nph- $\beta$ -D-saccharides and  $\beta$ -D-glucosides as substrates. The specificity is summarized in Table II in comparison with that of S $\beta$ -gly (7, 21).

Table II. Comparison of the kinetic parameters between his-tagged BGPh from *P. horikoshii* and Sβ-gly from *S. solfataricus* strain MT-4 against *p*-Nph-β-D-saccharides and β-D-glucosides.

Substrates	His-BGPh (90°C and pH 6.0)			Sβ-gly <sup>a</sup> (75°C and pH 6.5)		
	$k_{cat}$ (sec <sup>-1</sup> )	$K_m$ (mM)	$k_{cat} / K_m$ (mM <sup>-1</sup> sec <sup>-1</sup> )	$k_{cat}$ (sec <sup>-1</sup> )	$K_m$ (mM)	$k_{cat} / K_m$ (mM <sup>-1</sup> sec <sup>-1</sup> )
Laminaribiose	184	138.23	1.33	908	1.0	908.0
Cellobiose	194	1698.18	0.11	1333	30.0	44.4
Cellotriose	ND <sup>b</sup>	ND	ND	197	3.0	66
Cellotetraose	ND	ND	ND	584	1.7	343
β-Gentiobiose	ND	ND	ND	1360	100	14
<i>p</i> -Nph-β-D-Glcp	79	0.35	225.67	542	0.5	1084.0
<i>p</i> -Nph-β-D-Galp	123	1.30	94.34	1020	4.7	217.0
<i>p</i> -Nph-β-D-Xylp	3	0.10	31.83	284	4.0	71.0
<i>p</i> -Nph-β-D-Manp	2	0.14	14.60	NH <sup>c</sup>	NH	NH
Salicin	44	1.96	22.20	880	5.0	175.9
Methyl-β-D-Glcp (Alkyl : C <sub>1</sub> )	35	40.74	0.85	- <sup>d</sup>	-	-
n-Amyl-β-D-Glcp (Alkyl : C <sub>5</sub> )	31	2.02	15.11	256	1.1	232
n-Hexyl-β-D-Glcp (Alkyl : C <sub>6</sub> )	33	0.54	60.28	263	1.0	263
n-Octyl-β-D-Glcp (Alkyl : C <sub>8</sub> )	34	0.20	170.70	313	0.7	434
n-Nonyl-β-D-Glcp (Alkyl : C <sub>9</sub> )	39	0.08	471.57	-	-	-
n-Decyl-β-D-Glcp (Alkyl : C <sub>10</sub> )	37	0.08	469.62	-	-	-
n-Undecyl-β-D-Glcp (Alkyl:C <sub>11</sub> )	43	0.05	944.37	-	-	-
n-Dodecyl-β-D-Glcp (Alkyl:C <sub>12</sub> )	36	0.03	1152.90	-	-	-

<sup>a</sup> Cited from references (7, 21).

<sup>b</sup> ND; The parameters were not determined because of too high  $K_m$  values.

<sup>c</sup> NH; The substrate was not hydrolyzed by Sβ-gly.

<sup>d</sup> -; The parameters were not reported in the references.

His-BGPh hydrolyzed aryl glycosides efficiently, showing  $k_{cat}/K_m$  values decreasing in the order  $p\text{-Nph-}\beta\text{-D-Glcp} > p\text{-Nph-}\beta\text{-D-Galp} > p\text{-Nph-}\beta\text{-D-Xylp} > p\text{-Nph-}\beta\text{-D-Manp}$ . Beta-linked glucose dimers tested were poorly hydrolyzed; the order of preference was  $\beta\text{1-3} > \beta\text{1-4} > \beta\text{1-6}$ . The  $k_{cat}$  values of BGPh without His-tag for these  $\beta$ -linked glucose dimers approached  $400\text{ sec}^{-1}$ , which is comparable with those of  $S\beta\text{-gly}$  (Table II). His-BGPh probably had approximately 50% of the activity of BGPh due to interference by the His-tag located at the N-terminus, (Fig. 3). Surprisingly, the best substrates for His-BGPh were  $\beta\text{-D-glucosides}$  with long alkyl chains ( $\text{LA-}\beta\text{-D-Glcp}$ ). The  $K_m$  values decreased according to the elongation of the alkyl chain from  $C_1$  to  $C_{12}$ , although the  $k_{cat}$  value was constant (approximately  $35\text{ sec}^{-1}$ ) for each alkyl- $\beta\text{-D-Glcp}$ . The  $k_{cat}$  values of native type BGPh for  $\text{LA-}\beta\text{-D-Glcp}$  approached  $70\text{ sec}^{-1}$ , calculated on the basis of the value of His-BGPh, estimating a 50% decrease in the activity from the inhibitory effect of the His-tag. The value was also appreciable, around 30% of that of  $S\beta\text{-gly}$  (Table II). The  $K_m$  value of His-BGPh for the hydrolysis of  $n\text{-Dodecyl-}\beta\text{-D-Glcp}$  (alkyl chain :  $C_{12}$ ) was extremely low, 30 mM at  $90^\circ\text{C}$  and pH 6.0. Of the substrates examined thus far, the best substrate was  $n\text{-Dodecyl-}\beta\text{-D-Glcp}$  as shown in Table II. The  $k_{cat}/K_m$  value of His-BGPh against  $n\text{-Dodecyl-}\beta\text{-D-Glcp}$  was 5 times higher than that of  $p\text{-Nph-}\beta\text{-D-Glcp}$  and 870 times higher than that of laminaribiose. Even the value for  $n\text{-Octyl-}\beta\text{-D-Glcp}$  was 0.76 times higher than that of  $p\text{-Nph-}\beta\text{-D-Glcp}$  and 128 times higher than that of

laminaribiose. The  $k_{cat}/K_m$  value of  $S\beta$  - gly against n-Octyl- $\beta$  - D-Glcp, with the longest alkyl chain so far examined (21), was 0.4-fold higher than that for p-Nph- $\beta$  - D-Glcp and 0.48-fold higher than that for laminaribiose. Laminaribiose and cellobiose were not good substrates for the hydrolysis of His-BGPh because of their  $K_m$  values higher than 100 mM. His-BGPh also hydrolyzed cellotriose and cellotetraose with low efficiency: the kinetic parameters were not determined because of the extremely high  $K_m$  value, whereas  $S\beta$  - gly was able to hydrolyze these oligosaccharides with high efficiency: the  $k_{cat}/K_m$  values descended in the order; cellotetraose > cellotriose > cellobiose. Thus, the substrate specificity of His-BGPh is different from those of the other  $\beta$  - glycosidases, including  $S\beta$  - gly (7, 17, 21-23). BGPh has a novel substrate specificity with high efficiency to hydrolyze LA- $\beta$  - D-Glcp and low efficiency to hydrolyze any  $\beta$  - linked glucose dimer which is more hydrophilic than aryl- or alkyl- $\beta$  - D-Glcp. The subsite affinity ( $A_{(C11)}$ ) to bind a long alkyl chain ( $C_{11}$ ) was calculated according to the following equation;  $A_{(C11)} = RT((k_{cat}/K_m)_{\text{for n-Dodecyl-}\beta\text{-D-Glcp}} / (k_{cat}/K_m)_{\text{for Methyl-}\beta\text{-D-Glcp}})$ . The affinity was determined to be 4.26 kcal/mol. The value was reasonable when compared with the highest affinity (4.23 kcal/mol) known, that of the recognition of one glucose unit in the subsite structure of Saccharomycopsis amylase (13, 14). These facts indicate that the hydrophobicity of the aglycon part of the substrates is strongly recognized by the BGPh molecule and the hydrophobic substrates, including aryl- and LA-

$\beta$  - D-Glcp, are hydrolyzed effectively with low  $K_m$  values due to hydrophobic interaction between the aglycon moiety and the BGPh molecule. Thus, BGPh might be useful to synthesize novel  $\beta$  - glycosides, including new biosurfactants, using its transglycosylation activity because of its stability in organic solvents (data not shown).

Henrissat proposed an alternate and complementary classification scheme for glycosyl hydrolases based on amino acid sequence similarities (24-26). For example, glycosyl hydrolase family 1 is composed of exo-acting,  $\beta$  - specific enzymes with similar amino acid sequences. The five  $\beta$  - glycosidases, including BGPh from the archaea domain (as shown in Fig. 5), belong to family 1. Some family 1 glycosyl hydrolases also have glycosyl transferase activities. The *S. solfataricus*  $\beta$  - glucosidase has been implicated in the glycosylation of membrane lipid components (27). Similarly, the enzymatic analysis of BMPf predicted its possible role in the synthesis of intracellular components including protein, membrane components, or other compounds (17). Since the localization of BGPh on *E. coli* membrane strongly indicates the intimate interaction of the enzyme and lipid components, the detection of BGPh on the *Pyrococcus* cell surface using antibody against the enzyme must be done to clarify its true function in the *Pyrococcus* cell.

*The Structural Elements Responsible for Membrane Localization and the Conservation of Residues Forming the Active Site* - The sequence alignment among BGPh and four



different  $\beta$  - glycosidases, whose biochemical characteristics have been reported (7, 17, 21-23), is shown in Figure 5. According to the phylogenetic analysis based on the alignment, the tree has three branches : one corresponding to a  $\beta$  - glycosidase group that includes BGPf and S  $\beta$  - gly; another containing BMPf and BMPf, which were close to  $\beta$  - mannosidase. BGPh belongs to the third branch, located some distance from the first two branches. The polypeptide length of BGPh is also approximately 13% shorter than those of the other four  $\beta$  - glycosidases and might be one of the shortest sequences so far reported (8, 17, 18, 28). As shown in Figure 5, the residues E155 and H111 of BGPh correspond to E206 and H150 as the putative acid/base catalyst in the S  $\beta$  - gly molecule (28, 29), whose steric structure has been reported (30). The residues E324 and R75 of BGPh correspond to E387, the nucleophile, and R79 in the spatial proximity of the nucleophile (28, 29). The complex structure of *Bacillus polymixa*  $\beta$  - glycosidase with the inhibitor gluconate has been reported (31). The BGPh residues, Q19, H111, N154, E155, Y267, E324, W362, E369, and W370 are completely conserved (Fig. 5) and correspond to the *B. polymixa*  $\beta$  - glycosidase residues, Q20, H121, N165, E166, Y296, E352, W398, E405, and W406, which form the intimate interaction with the inhibitor (31).

To understand the localization mechanism of BGPh to the membrane, a major structural difference between BGPh and the other soluble  $\beta$  - glycosidases was analyzed using the sequence alignment and the steric structure of S  $\beta$  -

gly (30). The  $S\beta$  - gly molecule has the classic  $(\beta\alpha)_8$  barrel fold first seen in the structure of triose phosphate isomerase (32). For BGPh, the prominent deletion of more than 30 residues was found after the 78th residue, as indicated in Figure 5. The deletion region of BGPh corresponds to loops from the 89th to 125th residues of  $S\beta$  - gly, mainly shielding the helices 3 and 4 from solvent. The hydrophilic loops, which pack against the outer face of the barrel helices 3 and 4, were not present in the BGPh molecule. A tetrameric  $S\beta$  - gly structure has been reported, in which these loop regions were located at the four edges of regular tetragonal molecular arrangement (30). Figure 6 illustrates the location of the four hydrophilic edges and four hydrophobic areas which appear following the removal of the hydrophilic loops. Since BGPh as well as  $S\beta$  - gly was proved to be tetramer by gel filtration using buffer containing 0.01% Triton X-100 (data not shown), the deletion of these hydrophilic loops probably results in the exposure of helices 3 and 4 to the solvent at the four edges of the tetrameric structure. The exposed hydrophobic areas might interact with lipid components to embed the molecule in the membrane.

The increased hydrophobicity at barrel helices 3 and 4 is also indicated by the comparison of the hydropathy plots of BGPh and  $S\beta$  - gly, as shown in Figure 7. Two major hydrophobic clusters were observed in the region of BGPh between residues 79 and 210 corresponding to the region of  $S\beta$  - gly between residues 90 and 265. These residues form tertiary structures from the end of  $\beta$  - sheet 2 to the

beginning of  $\beta$  - sheet 5 of the  $(\beta\alpha)_8$  barrel fold (30). The first cluster was located between residues 79 to 114, forming a helix with a loop shortened by the deletion,  $\alpha$ -helix 2, and  $\beta$  - sheet 3. The second cluster was present between residues 131 and 210, corresponding the barrel fold between  $\alpha$ -helices 3 and 4 exposed to solvent. A hydrophilic module that might be important for enzyme orientation on the membrane was found between residues 114 to 131, corresponding to the hydrophilic helices at the molecule surface located between the  $\beta$  - strand and the  $\alpha$ -helix in the third repeat of the barrel fold. The two hydrophobic clusters, but not the hydrophilic module, were lacking in the corresponding region of S $\beta$  - gly (18).

A mechanism for the localization of BGPh is proposed here based on the possible hydrophobic interaction between the membrane and the exposed hydrophobic helices 3 and 4 at the four edges of the tetrameric structure exposed by the deletion of the hydrophilic loops. Furthermore, the mechanism is well supported by the hydropathy profile of BGPh, in which the hydrophobic cluster is formed by the barrel fold between  $\alpha$ -helices 3 and 4. The exposed hydrophobic areas may lead the hydrophobic substrates to the active site and bind them there. However, further studies using the crystallographic analysis are needed for a more definitive description of the detailed mechanism for recognition of the hydrophobic aglycon part, including a long alkyl-chain.

As described above, the present invention provides a novel  $\beta$  -glycosidase. The  $\beta$  -glycosidase is stable under

extreme conditions. Therefore, the  $\beta$ -glycosidase can be used to develop heterosaccharides with high optical purities.

#### REFERENCES

1. Cowan, D. A. (1992) The Archaeobacteria: Biochemistry and Biotechnology (Danson M. J., Hough, D. W., and Lunt, G.G., eds.) pp. 149-170. Portland Press, London and Chapel Hill
2. Tricone, A., Nicolaus, B., Lama, L., and Gambacorta, A. (1993) Indian J. Chem.. (Section B) 32, 25-29
3. Hudson, R. C., Schofield, L. R., Coolbear, T., Daniel, R. M., and Morgan, H. W. (1991) Biochem. J. 273, 645-650
4. Kume, S. and Fujio, Y. (1991) J. Gen. Appl. Microbiol. 37, 25-34
5. Tokatlidis, K., Dhurjati, P., Millet, J., Beguin, P., and Rossi, M. (1991) FEBS Lett. 282, 205-208
6. Toone, E. J., Simon, E. S., Bednarski, M. D., and Whitesides, G. M. (1989) Tetrahedron 45, 5365-5422
7. Tricone, A., Improta, R., Nucci, R., Rossi, M., and Gambacorta, A. (1994) Biocatalysis 10, 195-210
8. Kwarabayasi, Y., Sawada, M., Horikoshi, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Yoshizawa, T., Nakamura, Y., Robb, F. T., Horikoshi, K., Masuchi, Y., Shizuya, H., and Kikuchi, H. (1998) DNA Res. 5, 55-76

9. Kawarabayasi, Y., Sawada, M., Horikoshi, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Yoshizawa, T., Nakamura, Y., Robb, F. T., Horikoshi, K., Masuchi, Y., Shizuya, H., and Kikuchi, H. (1998) DNA Res. 5, 147-155
10. Kaneko, T., Tanaka, A., Sato, S., Kotani, H., Sazuka, T., Miyajima, N., Sugiura, M., and Tabata, S. (1995) DNA Res. 2, 153-166
11. Laemmli, U. K. (1970) Nature 227, 680-682
12. Sakada, M. and Hiromi, K. (1976) J. Biochem. 80, 547-555
13. Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S., and Honda, K. (1991) J. Biochem. 109, 566-569
14. Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S., and Honda, K. (1992) FEBS Lett. 310, 216-218
15. Sugauma, T., Matsuno, R., Ohnishi, M., and Hiromi, K. (1978) J. Biochem. 84, 293-316
16. Hiromi, K. (1970) Biochem. Biophys. Res. Commun. 40, 1-6
17. Bauer, M. W., Bylina, E. J., Swanson, R. V., and Kelly, R. M. (1996) J. Biol. Chem. 271, 23749-23755
18. Cubellis, M. W., Rozzo, C., Montecucchi, P., and Rossi, M. (1990) Gene 94, 89-94
19. Nei M. (1987) Molecular Evolutionary Genetics, pp. 293-298. Columbia University Press, New York, N.Y.
20. Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132

21. Nucci, R., Moracci, M., Vaccaro, C., Vespa, N., and Rossi, M. (1993) *Biotechnol. Appl. Biochem.* 17, 239-250
22. Kengen, S. W. M., Luesink, E. J., Stams, A. J. M., and Zehnder, A. J. B. (1993) *Eur. J. Biochem.* 213, 305-312
23. Bauer, M. W. and Kelly, R. M. (1998) *Biochemistry* 37, 17170-17178
24. Henrissat, B. (1991) *Biochem. J.* 280, 309-316
25. Henrissat, B. and Bairoch, A. (1993) *Biochem. J.* 293, 781-788
26. Henrissat, B. and Bairoch, A. (1996) *Biochem. J.* 316, 695-696
27. Grogan, D. W. (1991) *Appl. Environ. Microbiol.* 57, 1644-1649
28. Febbraio, F., Barone, R., D'Auria, S., Rocci, M., and Nucci, R. (1997) *Biochemistry* 36, 3068-3075
29. Moracci, M., Trincone, A., Perugino, G., Ciaramella, M., and Rossi, M. (1998) *Biochemistry* 37, 17262-17270
30. Aguilar, C. F., Sanderson, I., Moracci, M., Ciaramella, M., Nucci, R., Rocci, M., and Pearl, L. H. (1997) *J. Mol. Biol.* 271, 789-802
31. Sanz-Aparicio, J., Hermoso, J. A., Martinez-Ripollo, M., Lequerica, J. L., and Polaina, J. (1998) *J. Mol. Biol.* 275, 491-502
32. Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D., and Waley, S. G. (1975) *Nature* 255, 609-614

All publications, patents and patent applications

cited herein are incorporated herein by reference in their entity.

The invention has been described in detail with reference to various embodiments, and it will now be apparent from the foregoing to those skilled in the art that changes and modifications may be made without departing from the invention in its broader aspects, and it is the invention, therefore, in the appended claims to cover all such changes and modifications as fall within the true spirit of the invention.

The following is information on sequences described herein:

#### SEQUENCE LISTING

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 Asp Ile Ser Glu Arg Lys Thr Gln Met Gly Trp Ser Val Tyr Pro Lys  
                     290                      295                      300  
 Gly Ile Tyr Met Ala Leu Lys Lys Ala Ser Arg Tyr Gly Arg Pro Leu  
 305                      310                      315                      320  
 Tyr Ile Thr Glu Asn Gly Ile Ala Thr Leu Asp Asp Glu Trp Arg Val  
                     325                      330                      335  
 Glu Phe Ile Ile Gln His Leu Gln Tyr Val His Lys Ala Ile Glu Asp  
                     340                      345                      350  
 Gly Leu Asp Val Arg Gly Tyr Phe Tyr Trp Ser Phe Met Asp Asn Tyr  
                     355                      360                      365  
 Glu Trp Lys Glu Gly Phe Gly Pro Arg Phe Gly Leu Val Glu Val Asp

370                      375                      380  
 Tyr Gln Thr Phe Glu Arg Arg Pro Arg Lys Ser Ala Tyr Val Tyr Gly  
 385                      390                      395                      400  
 Glu Ile Ala Arg Ser Lys Glu Ile Lys Asp Glu Leu Leu Lys Arg Tyr  
                     405                      410                      415  
 Gly Leu Pro Glu Leu Gln Leu  
                     420

<210> 3

<211> 57

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: An upper primer designed to  
 create  
 the NdeI site.

<400> 3

taagaaggag atatacatat gccgctgaaa ttcccggaaa tgtttctcctt tggtacc 57

<210> 4

<211> 46

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: A lower primer designed to  
create the BamHI site.

<400> 4

tttactgcag agaggatccc taatcctaaa gttgaagttc tggtag

46

WHAT IS CLAIMED IS:

1. A thermophilic enzyme having  $\beta$ -glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added.

2. The enzyme of claim 1, having an optimum temperature of 100° C or higher.

3. A DNA which is capable of hybridizing to the nucleotide sequence of SEQ ID NO: 1 or to the complement thereof under such conditions that the hybridization is carried out in 6xSSC and 50% formamide at 42 °C and the washing process is carried out in 6xSSC and 40% formamide at 25 °C, and which encodes a thermophilic enzyme having  $\beta$ -glycosidase activity.

4. The DNA of claim 3, which encodes the enzyme of claim 1.

5. A recombinant vector containing the DNA of claim 3 therein.

6. A host cell transformed with the recombinant vector of claim 5.

7. A process for producing the enzyme of claim 1, comprising culturing a host cell transformed with an

expression vector containing a DNA encoding the enzyme and then collecting the enzyme from the resultant culture.

8. A process for the hydrolysis of a  $\beta$ -glycoside having a long alkyl chain at the reducing end, with a thermophilic enzyme having  $\beta$ -glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added.

9. The process of claim 8, wherein the long alkyl chain is an alkyl group having carbon atoms of 8 or more.

10. The process of claim 8, wherein the hydrolysis is carried out at a temperature of 85° C or higher.

11. The process of claim 8, wherein the hydrolysis is carried out at a temperature of 100° C or higher.



# ABSTRACT OF THE DISCLOSURE

The invention relates to a thermophilic enzyme having  $\beta$ -glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added.

FIG.1

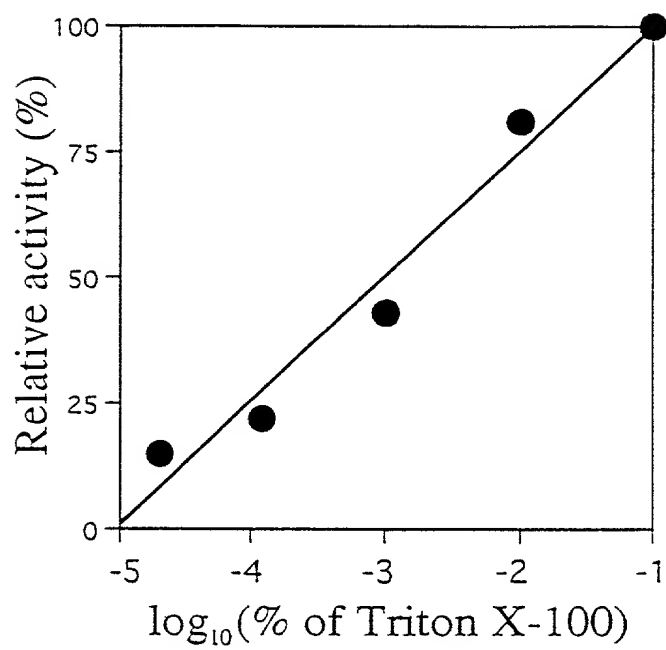


FIG.2

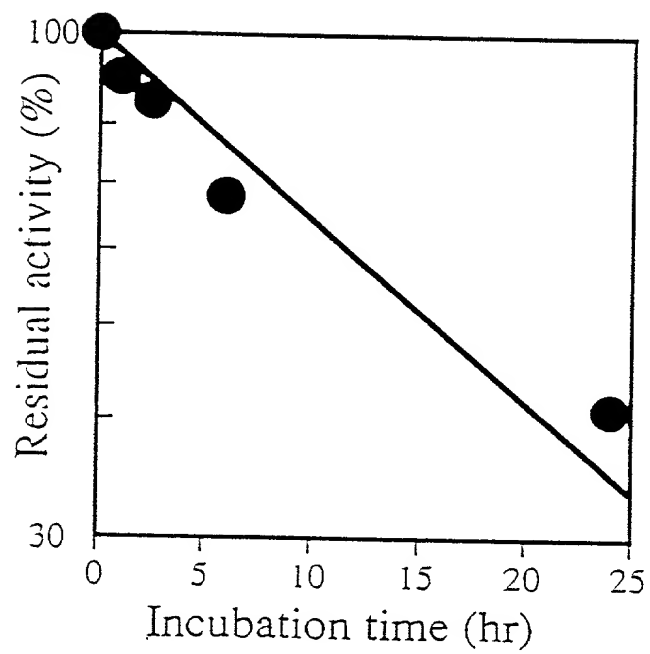


FIG.3

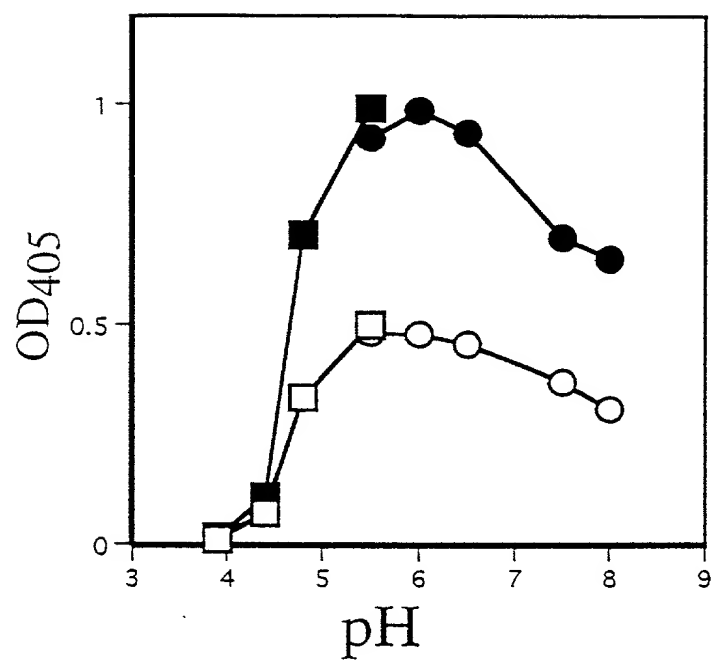


FIG.4

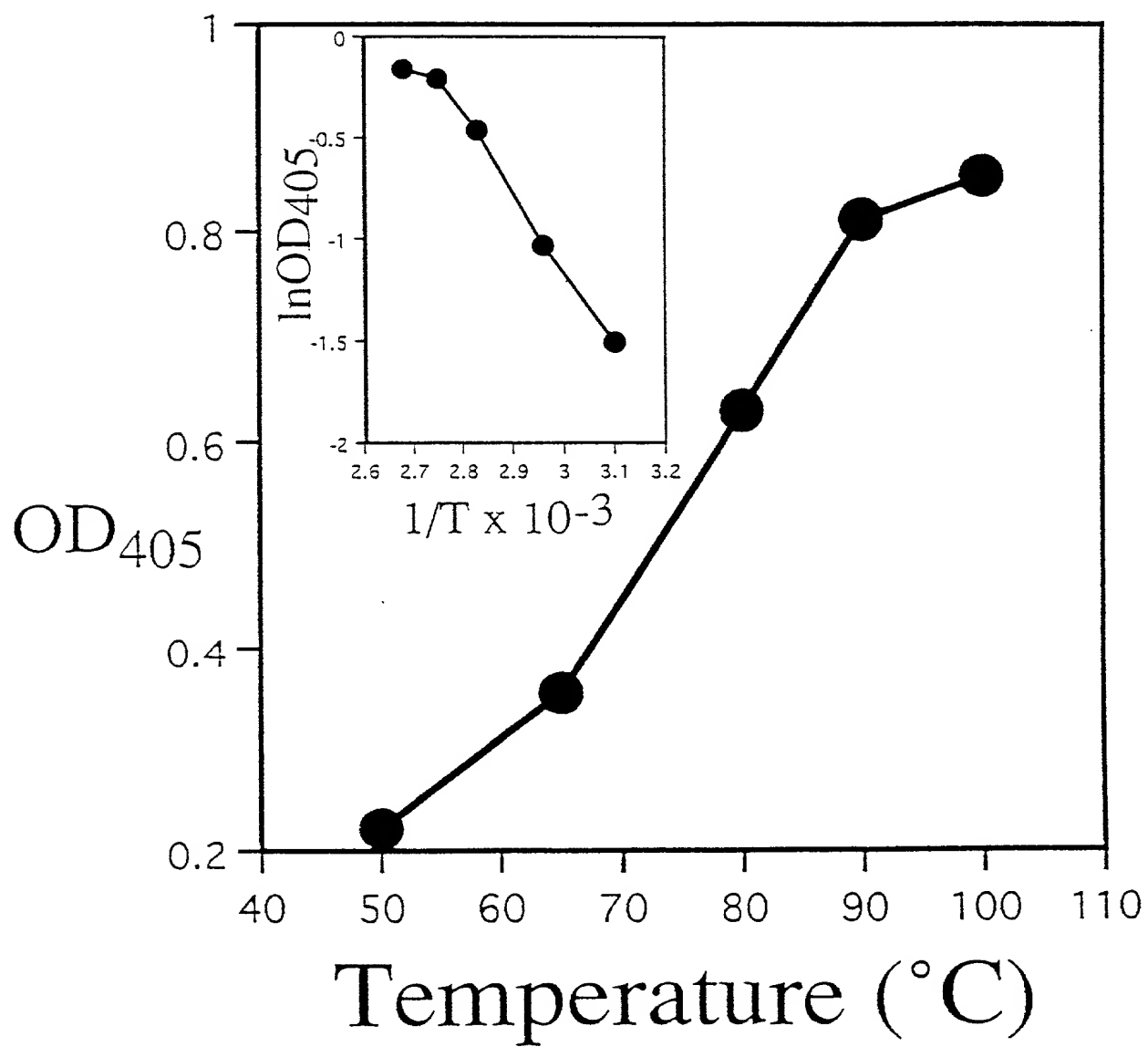


FIG.5

BG Ph	MPLKFPENFL	ELCATSSGHC	EDNNRW----	---KDWVYVE	Q-----IGK	37
BM Ph	M-----HFV	WGVVQSAFCF	EMGOPYRRNI	OPRSOWVYVW	ROPVNIKNOL	44
BM Pf	M---FPEHFL	WQVAQSGFCF	EMGDKLRNI	OTNTDWHHWV	ROKTNIEKGL	47
BG Pf	M--KFPKNEF	EGYSWSGFCF	EMGLP--GSE	-VESDWWVWV	HOKENIASGL	45
Sb-gly	M-YSFNPFER	EGWSQAGFCF	EMGTP--GSE	OPNTDWKVWV	HOPENMAAGL	47
Consensus	M--.FP.EL	ELCATSSGHC	EMG.P.---	D...DWV.WV	.D..NI..GL	50
BG Ph	LPYRSG-KAC	NHWELYRDDI	QLMTSLGANA	YRFSIEWERI	FF-----	78
BM Ph	VSGDLPEEGI	NNYELVEIDH	RLAKELCLNA	YQLTIEWERI	FFCPTFNVEV	94
BM Pf	VSGDLPEEGI	NNYELVEKDH	ELARKLGLNA	YRIGIEWERI	FFWPTFFIDV	97
BG Pf	VSGDLPENGP	AYWHLYKQDH	DIAEKLGMDC	IRGGIEWARI	FFKPTFDVKV	95
Sb-gly	VSGDLPENGP	GYWGNVYKTFH	DNAQKYGCKI	ARLNIEWERI	FFNPLPRPQN	97
Consensus	VSGDLPE.G.	N.WELV..DH	..A.KLCLNA	YR..IEWERI	FF.PT....V	100
BG Ph	-----EENK	FRF-----	DAFMKIREII	DLLLTFCITP		105
BM Ph	EFERD-GYGL	IKKVKIEKEH	LEELDKLANQ	KEVRHYLANV	RNLKKGFTT	143
BM Pf	DYSYNESYNL	IEDVKITKDT	LEELDEIANK	REVAYYRSVI	NSLRSGGFKV	147
BG Pf	DVEKD-EEGN	IISVDVPEST	IKLEKIANM	EALHYRKIY	SDWKEPCKTF	144
Sb-gly	FDESKQDVTE	VEINENELKR	LDK---ANK	DALNHYREIF	KDLKSPGLYF	144
Consensus	..E...I..V..E..	L..EL...AN.	..A..HYR..I.	..LK..FC..T.		150
BG Ph	LVTLHFTSP	LMF-----	-----MKKGG	FLREENLKHV	EKYIEKVA-E	142
BM Ph	FVTLNHQTNP	IMLHDPLETR	GNFQKARAFG	WVDERTIIEF	AKYAYVAWK	193
BM Pf	IVNLNHFETLP	IMLHDPLEAR	ERALTNKRAG	WNPRTVIEF	AKYAYIAWK	197
BG Pf	ILNLNHWPLP	IMLHDPPIAVR	KLGPDRAPAG	WLDEKTVVEF	VKFAAFVAKH	194
Sb-gly	ILNLNHWPLP	IMLHDPPIVR	R-GDFTGPSG	WLSTRTVYEF	ARFSAYIAWK	193
Consensus	IVNL..EL..TLP	IM..HDPI..R	.....G..ML	ERTV..EF	AKYAYVA..K	200
BG Ph	LLEKVKLVAT	FNEFNVKMM	GYLTAY--WP	FFIRSPFKAF	KVAANLLIAH	190
BM Ph	FDMVVDWST	FDEEMVITAEI	GYLAPYVGWP	PGILNPSAAK	KVIINQIVAH	243
BM Pf	FGDIVDMWST	FNEFNVVVEL	GYLAPYSGFP	PGVLNPSAAK	LAILHMINAH	247
BG Pf	LDDIVDMWST	MNEFNVVYNQ	GYINLRSQFP	PGYLSFEAAE	KAKFNLIQAH	244
Sb-gly	FDDIVDEYST	MNEFNVVGGI	GYVGKSGFP	PGYLSFELSR	RHMVNIICAH	243
Consensus	FDD..MD..WST	FNEFNV..L	GYL..YSGFP	PG..LSPEAA.	K...N..I..AH	250
BG Ph	ALAYELLHG-	-----KF	K--VGIVKN	I--PIILPAS	DKERDRKAE	226
BM Ph	APAYDSIKKF	-----SS	KP--VGITLN	II--PAYPLOP	NDSKSVRAAE	282
BM Pf	ALAYRQIKKF	DTEKADKDSK	EPAEVGIIYN	NIGVAYPKDP	NDSKDVKAEE	297
BG Pf	ICAYDAIKEY	-----SE	KS--VGVIYA	F--AWH--DP	LAEEYKDEVE	280
Sb-gly	APAYDKIKSV	-----SK	KP--VGIIYA	N--SSF--QP	LTDKDMEAIE	279
Consensus	A..AYD..IK..	-----S.	KP--VGIIYN	..-----DP	...KD...AAE	300
BG Ph	KADNLFNWHF	LDAIWSGKYR	GVF---KTYR	IP-QSDACFI	GANNYTASEV	272
BM Ph	NYDLFHNRLF	LEAVNRGNVD	LDITGE-YTK	IPHIKRNDAI	GANNYTREV	331
BM Pf	NDNFFHSGLF	FEAIHKGKLN	IEFDGETFID	APYLKGNDAI	GANNYTREV	347
BG Pf	EIRKGD---	YE-FV-----	-----TILH	S--KGKLDIAI	GANNYSRLVY	312
Sb-gly	MAENDNRWWF	FDALIRGEIT	RGN--EKIVR	DOLKGRIDA	GANNYTITV	327
Consensus	.....F..EAI..G...	....E....	..P....DAI	GANNYTREV		350
BG Ph	RHT---WNPL	K-----	FFFEVK---L	ADISERKTQM	GWEVVEKGLY	307
BM Ph	KYVEPKYEEL	PLITFVGVEG	YGYSGNPNSL	SPDNNPTSDF	GWEVVEKGLY	381
BM Pf	TYQEPMPFPI	PLITFKGVQG	YGYACRPRTL	SKDDRPVSDI	GWEVVEKGLY	397
BG Pf	GAKDGLHVL	P-----G	YGFMSERGGF	AKSGRPASDF	GWEVVEKGLY	354
Sb-gly	KRTEKGYVSL	G-----G	YGHGCERNVS	SLAGLPTSDF	GWEVVEKGLY	369
Consensus	...E.....L	P-----G	YG.....L	S....P.SDF	GWEVVEKGLY	400
BG Ph	MALKKA-SFY	GRPLYITENG	DAITLDEWRV	EFIICHLQYV	HKAIEICLGV	356
BM Ph	OSTLEA-AEY	NKEVFITENG	DADSKDILRP	RYTIIDHNEV	KKLIENGIMV	430
BM Pf	DSIVEA-HMY	GVPVYITENG	DADSKDILRP	YYIASHIKMI	EKAFCIGYEV	446
BG Pf	NLLKYLNNFY	ELPMIITENG	DADADRYLRP	HYLVSHLKAV	YNAMKEGACV	404
Sb-gly	DVLTKYWNFY	HLVYVITENG	DADADRYLRP	YYLVSHVYQV	HRAINSACLV	419
Consensus	D..L..A-..Y	..P.YITENG	DAD..D..LRP	..YI..SH...V	..KAIE..G..LV	450
BG Ph	FGYFHWSEMD	NMEWKEGFCP	RFGLYEVDYQ	TFERRERKSA	Y-VYGEIARS	405
BM Ph	GGYFHWALTD	NMEWAGFKFI	RFGLYEVDLI	TKERIERRRS	VEIYKHIUME	480
BM Pf	FGYFHWALTD	NMEWALGFRM	RFGLYEVDLI	TKERIERREKS	VSIFREIIVAN	496
BG Pf	FGYFHWALTD	NMEWAGGFRM	RFGLYEVDYQ	TKKRYLRPSA	L-VFREIATQ	453
Sb-gly	FGYFHWALTD	NMEWAGGFSM	RFGLYEVDYN	TKRLYLRPSA	L-VYREIATN	468
Consensus	FGYFHWALTD	NMEWAGGFSM	RFGLYEVDYN	TKR..LR..SA	..-VYREIA..	500
BG Ph	KEIKDELLKR	YGL---PELQ	L			423
BM Ph	-GIE-----	-----				483
BM Pf	NGVTKKIEE-	-----ELLR	G			510
BG Pf	KEIPEELAH	AOLKFVT--R	K			472
Sb-gly	GAITDEIEHL	NSVPPVKPLR	H			489
Consensus	..I..E....	.....LR				521

FIG.6

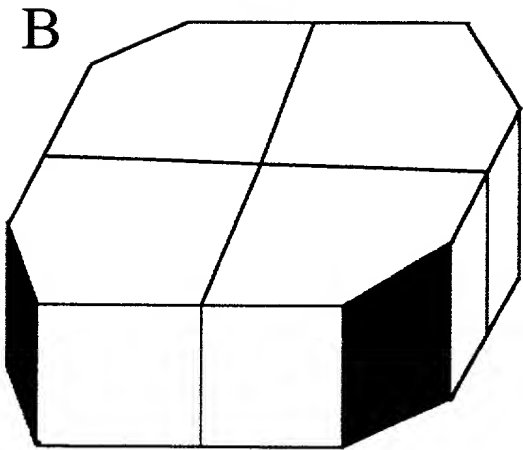
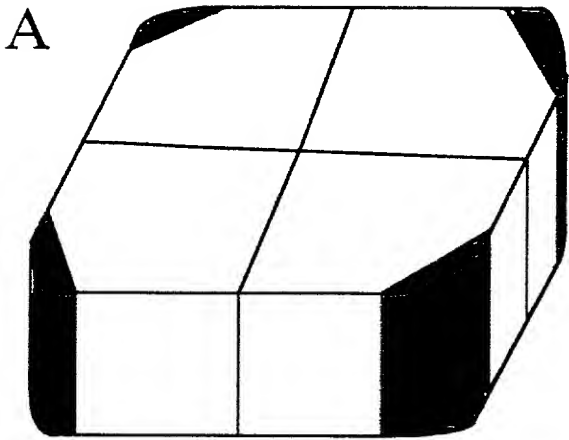
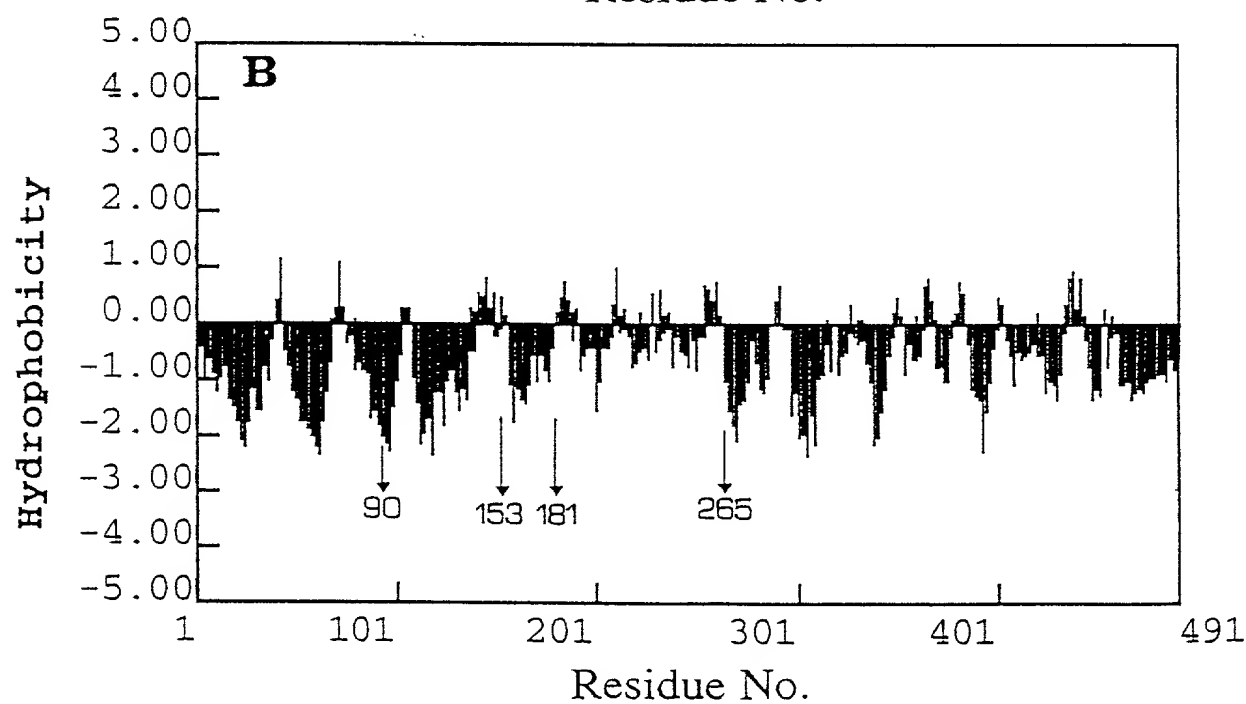
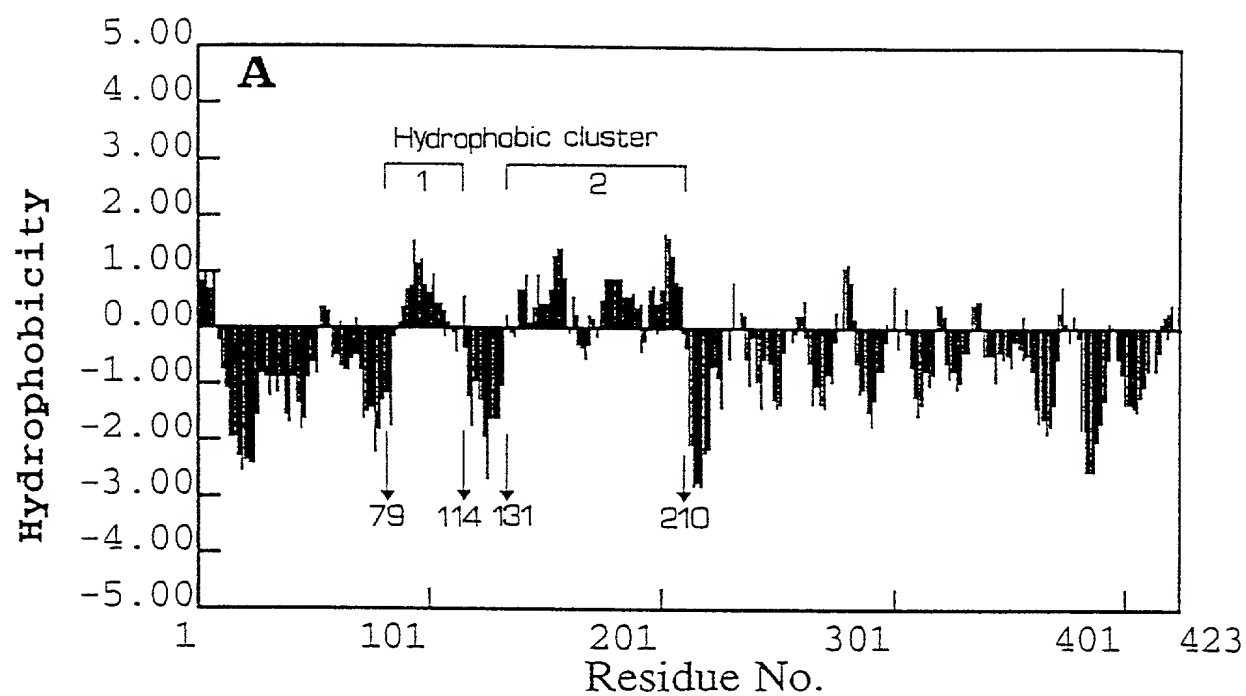


FIG.7





# SEQUENCE LISTING

<110> Director-General of Agency of Industrial Science and Technology

<120> Heat-resistant enzyme having  $\beta$  - glycosidase activity

<130> PH-679US

<150> JP 10-222866

<141> 1998-08-06

<160> 4

<170> PatentIn Ver. 2.0

<210> 1

<211> 1269

<212> DNA

<213> Pyrococcus horikoshii

<220>

<221> CDS

<222> (1) .. (1269)

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Met Pro Leu Lys Phe Pro Glu Met Phe Leu Phe Gly Thr Ala Thr Ser

1

5

10

15

tcc cat cag ata gag gga aat aat aga tgg aat gat tgg tgg tac tat 96

Ser His Gln Ile Glu Gly Asn Asn Arg Trp Asn Asp Trp Trp Tyr Tyr	
20 25 30	
gag cag att gga aag ctc ccc tac aga tct ggt aag gct tgc aat cac	144
Glu Gln Ile Gly Lys Leu Pro Tyr Arg Ser Gly Lys Ala Cys Asn His	
35 40 45	
tgg gaa ctt tac agg gat gat att cag cta atg acc agc ttg ggc tat	192
Trp Glu Leu Tyr Arg Asp Asp Ile Gln Leu Met Thr Ser Leu Gly Tyr	
50 55 60	
aat gct tat agg ttc tcc ata gag tgg agc agg cta ttc cca gag gaa	240
Asn Ala Tyr Arg Phe Ser Ile Glu Trp Ser Arg Leu Phe Pro Glu Glu	
65 70 75 80	
aat aaa ttt aat gaa gat gct ttc atg aaa tac cgg gag att ata gac	288
Asn Lys Phe Asn Glu Asp Ala Phe Met Lys Tyr Arg Glu Ile Ile Asp	
85 90 95	
ttg tta ttg acg aga ggt ata act ccc ctg gtg acc cta cac cac ttt	336
Leu Leu Leu Thr Arg Gly Ile Thr Pro Leu Val Thr Leu His His Phe	
100 105 110	
act agc cct ctc tgg ttc atg aag aaa ggt ggc ttc ctt agg gag gag	384
Thr Ser Pro Leu Trp Phe Met Lys Lys Gly Gly Phe Leu Arg Glu Glu	
115 120 125	
aac cta aaa cat tgg gaa aag tac ata gaa aag gtt gct gag ctt tta	432
Asn Leu Lys His Trp Glu Lys Tyr Ile Glu Lys Val Ala Glu Leu Leu	
130 135 140	
gaa aaa gtt aaa cta gta gct acc ttc aat gag ccg atg gta tac gta	480
Glu Lys Val Lys Leu Val Ala Thr Phe Asn Glu Pro Met Val Tyr Val	
145 150 155 160	
atg atg gga tat cta acg gct tat tgg ccc cca ttc att agg agt cca	528
Met Met Gly Tyr Leu Thr Ala Tyr Trp Pro Pro Phe Ile Arg Ser Pro	
165 170 175	

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 Phe Lys Ala Phe Lys Val Ala Ala Asn Leu Leu Lys Ala His Ala Ile  
 180 185 190  
 gcc tat gaa ctt ctt cat ggg aaa ttc aaa gtt gga atc gta aag aat 624  
 Ala Tyr Glu Leu Leu His Gly Lys Phe Lys Val Gly Ile Val Lys Asn  
 195 200 205  
 att ccc ata ata ctc cca gcg agt gac aag gag agg gat aga aaa gcc 672  
 Ile Pro Ile Ile Leu Pro Ala Ser Asp Lys Glu Arg Asp Arg Lys Ala  
 210 215 220  
 gct gag aaa gct gat aat tta ttt aac tgg cac ttt ttg gat gcg ata 720  
 Ala Glu Lys Ala Asp Asn Leu Phe Asn Trp His Phe Leu Asp Ala Ile  
 225 230 235 240  
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 Trp Ser Gly Lys Tyr Arg Gly Val Phe Lys Thr Tyr Arg Ile Pro Gln  
 245 250 255  
 agt gac gca gat ttc att ggg gtt aac tat tac acg gcc agc gaa gta 816  
 Ser Asp Ala Asp Phe Ile Gly Val Asn Tyr Tyr Thr Ala Ser Glu Val  
 260 265 270  
 agg cat act tgg aat cct tta aaa ttc ttc ttt gag gtg aaa tta gcg 864  
 Arg His Thr Trp Asn Pro Leu Lys Phe Phe Phe Glu Val Lys Leu Ala  
 275 280 285  
 gat att agc gag agg aag act caa atg gga tgg agc gtt tat cca aaa 912  
 Asp Ile Ser Glu Arg Lys Thr Gln Met Gly Trp Ser Val Tyr Pro Lys  
 290 295 300  
 gga ata tac atg gcc ctt aaa aaa gct tcc agg tat gga agg cct ctt 960  
 Gly Ile Tyr Met Ala Leu Lys Lys Ala Ser Arg Tyr Gly Arg Pro Leu  
 305 310 315 320  
 tat att acg gaa aac gga ata gcg acg ctt gat gat gaa tgg aga gtg 1008  
 Tyr Ile Thr Glu Asn Gly Ile Ala Thr Leu Asp Asp Glu Trp Arg Val

325                      330                      335  
 gaa ttc ata att caa cac ctc caa tac gtt cat aag gct atc gaa gac 1056  
 Glu Phe Ile Ile Gln His Leu Gln Tyr Val His Lys Ala Ile Glu Asp  
 340                      345                      350  
 ggc ctg gat gta aga ggt tac ttc tat tgg tca ttt atg gat aac tac 1104  
 Gly Leu Asp Val Arg Gly Tyr Phe Tyr Trp Ser Phe Met Asp Asn Tyr  
 355                      360                      365  
 gag tgg aaa gag ggg ttt ggg cct aga ttt ggc cta gtg gaa gtt gat 1152  
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 Tyr Gln Thr Phe Glu Arg Arg Pro Arg Lys Ser Ala Tyr Val Tyr Gly  
 385                      390                      395                      400  
 gaa att gca aga agt aag gaa ata aag gat gag cta tta aag aga tat 1248  
 Glu Ile Ala Arg Ser Lys Glu Ile Lys Asp Glu Leu Leu Lys Arg Tyr  
 405                      410                      415  
 ggc cta cca gaa ctt caa ctt 1269  
 Gly Leu Pro Glu Leu Gln Leu  
 420

<210> 2

<211> 423

<212> PRT

<213> Pyrococcus horikoshii

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1

5

10

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 20 25 30  
 Glu Gln Ile Gly Lys Leu Pro Tyr Arg Ser Gly Lys Ala Cys Asn His  
 35 40 45  
 Trp Glu Leu Tyr Arg Asp Asp Ile Gln Leu Met Thr Ser Leu Gly Tyr  
 50 55 60  
 Asn Ala Tyr Arg Phe Ser Ile Glu Trp Ser Arg Leu Phe Pro Glu Glu  
 65 70 75 80  
 Asn Lys Phe Asn Glu Asp Ala Phe Met Lys Tyr Arg Glu Ile Ile Asp  
 85 90 95  
 Leu Leu Leu Thr Arg Gly Ile Thr Pro Leu Val Thr Leu His His Phe  
 100 105 110  
 Thr Ser Pro Leu Trp Phe Met Lys Lys Gly Gly Phe Leu Arg Glu Glu  
 115 120 125  
 Asn Leu Lys His Trp Glu Lys Tyr Ile Glu Lys Val Ala Glu Leu Leu  
 130 135 140  
 Glu Lys Val Lys Leu Val Ala Thr Phe Asn Glu Pro Met Val Tyr Val  
 145 150 155 160  
 Met Met Gly Tyr Leu Thr Ala Tyr Trp Pro Pro Phe Ile Arg Ser Pro  
 165 170 175  
 Phe Lys Ala Phe Lys Val Ala Ala Asn Leu Leu Lys Ala His Ala Ile  
 180 185 190  
 Ala Tyr Glu Leu Leu His Gly Lys Phe Lys Val Gly Ile Val Lys Asn  
 195 200 205  
 Ile Pro Ile Ile Leu Pro Ala Ser Asp Lys Glu Arg Asp Arg Lys Ala  
 210 215 220  
 Ala Glu Lys Ala Asp Asn Leu Phe Asn Trp His Phe Leu Asp Ala Ile  
 225 230 235 240  
 Trp Ser Gly Lys Tyr Arg Gly Val Phe Lys Thr Tyr Arg Ile Pro Gln

245	250	255	
Ser Asp Ala Asp Phe Ile Gly Val Asn Tyr Tyr Thr Ala Ser Glu Val			
260	265	270	
Arg His Thr Trp Asn Pro Leu Lys Phe Phe Phe Glu Val Lys Leu Ala			
275	280	285	
Asp Ile Ser Glu Arg Lys Thr Gln Met Gly Trp Ser Val Tyr Pro Lys			
290	295	300	
Gly Ile Tyr Met Ala Leu Lys Lys Ala Ser Arg Tyr Gly Arg Pro Leu			
305	310	315	320
Tyr Ile Thr Glu Asn Gly Ile Ala Thr Leu Asp Asp Glu Trp Arg Val			
325	330	335	
Glu Phe Ile Ile Gln His Leu Gln Tyr Val His Lys Ala Ile Glu Asp			
340	345	350	
Gly Leu Asp Val Arg Gly Tyr Phe Tyr Trp Ser Phe Met Asp Asn Tyr			
355	360	365	
Glu Trp Lys Glu Gly Phe Gly Pro Arg Phe Gly Leu Val Glu Val Asp			
370	375	380	
Tyr Gln Thr Phe Glu Arg Arg Pro Arg Lys Ser Ala Tyr Val Tyr Gly			
385	390	395	400
Glu Ile Ala Arg Ser Lys Glu Ile Lys Asp Glu Leu Leu Lys Arg Tyr			
405	410	415	
Gly Leu Pro Glu Leu Gln Leu			
420			

<210> 3

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: An upper primer designed to  
create  
the NdeI site.

<400> 3

taagaaggag atatacatat gccgctgaaa ttcccgaaa tgtttctctt tggtagc 57

<210> 4

<211> 46

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: A lower primer designed to  
create the BamHI site.

<400> 4

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